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SPECIAL NOTE

This study is scheduled to expire on 2/28/2011 however we have not yet completed several studies which we feel are critical to accomplishing the specific aims of this project. As such, a no cost extension was filed with the DOD. Additionally a modified SOW was submitted with this NCE, as there have been some deviations from our original SOW. We are submitting this annual report in anticipation of receiving approval of our NCE.

INTRODUCTION

Androgen ablation, or androgen deprivation therapy (ADT), is the mainstay of treatment for patients with locally advanced or metastatic prostate cancer. This therapy is only temporizing, however, and within 3-4 years the vast majority of patients develop androgen independent prostate cancer (AIPC). Once a patient develops AIPC, treatment options are limited and less effective, with first line treatment providing only 13-15 month survival. This seminal event in disease progression has been the subject of much research, and while many factors are likely to be involved, the androgen receptor (AR) has been found to play a pivotal role in both the development and maintenance of AIPC. Many changes in the AR have been described, including mutation, constitutive activation and changes in expression levels.

Like all receptors, AR's tertiary structure is critical to its function. The chaperone proteins heat shock protein-90 (Hsp90) and Hsp40 are necessary for the correct formation of AR's tertiary structure. Subsequently a second co-chaperone, C-terminal Hsp interacting protein (CHIP), was characterized and found to bind HSP 70 and 90. CHIP contains E3 ligase activity which targets proteins for proteosomal ubiquitination. CHIP also binds directly to a highly conserved portion of AR which increases AR degradation. In cells where CHIP is overexpressed, AR synthesis is decreased and much of the AR produced has a defective tertiary structure. The addition of proteosomal inhibitors to the cells, does not restore AR levels to normal, indicating that AR degradation/suppression is occurring by non-proteosomal pathway(s) as well.

BODY

In the first studies of this experiment, we found that overexpression of CHIP in four prostate cancer cell lines (LNCaP, LNCaP-Tsai, C4-2, and PC-3) reduced AR expression in all of the AR expressing cells. A hormone binding assay then demonstrated that, not only was AR expression decreased, but ligand binding was decreased as well, both in the presence and absence of dihydrotestosterone (DHT). CHIP also had an effect on prostate specific antigen (PSA) production. PSA is an androgen sensitive serine protease made by both LNCaP and C4-2b cells. In cells overexpressing CHIP, both absolute and cell-count corrected concentrations of PSA were reduced.

But not only did CHIP overexpression reduce AR expression, ligand binding and PSA production also had an impact on cell growth. In AR expressing cell lines (C4-2b, LNCaP, and LNCaP-Tsai), CHIP overexpression decreased the growth and proliferation of these cell lines. When the growth cycle of these cells was analyzed, the hormone sensitive LNCaP cells exhibited evidence of growth arrest, while the androgen independent cell lines died. Annexin V analysis suggested that these two AR expressing hormone refractory prostate cancer cell lines died via autophagy and not apoptosis.

Because of these unexpected findings, we departed from our original SOW, and sought to identify the downstream targets of CHIP via Affymetrix array. Our high throughput analysis identified several genes that appeared to be either upregulated or downregulated by CHIP overexpression in either LNCaP (hormone sensitive) or C4-2b and LNCaP-Tsai (hormone refractory) cells. These genes, which included RhoE, SenP1, ARC, SASH1, Edg4, ACVR1, and APPL1 were reported in both the 2007 and 2008 annual reports. Confirmatory RT-PCR studies demonstrated that SenP1 levels were reduced by CHIP overexpression in hormone refractory cells (C4-2 and LNCaP Tsai) and increased in hormone sensitive cells (LNCaP).

SENP1 is an enzyme involved in the SUMOylation pathway. This pathway was first described in 1996-1997, when a new ubiquitin-like protein was characterized.[1] Unlike ubiquitination, however, sumoylation does not target proteins for degradation. Instead, sumoylation appears to stabilize proteins or alter their localization, function, or degree of function. Interestingly, SUMO proteins can act concomitantly or compete with ubiquitin. Since its characterization, a number of proteins have been identified as sumoylation substrates, including androgen receptor and p53.[1,2]

Reversal of sumoylation is carried out by a family of proteases known as SENP's. In a recent study by Cheng and colleagues, SENP1 produced a ligand-dependent, 23-fold increase in AR's transcriptional activity in LNCaP cells.[1] This effect could not be produced by any of the other members of the SENP family. When the sumoylation sites on AR were mutated, SENP1 still had the same effect on transcription, which suggests that SENP1's impact on AR is not via direct sumoylation of the receptor.

In last year's annual report, we documented several findings. CHIP overexpression did not appear to significantly impact cell cycle markers Cyclin E1 or Cyclin D1. Given the lack of significant change in Cyclin E1 and Cyclin D1, we redirected our efforts to examine the effect of androgen withdrawal on SenP1 expression in LNCaP, C4-2b and LNCaP-Tsai cells. At baseline, there are only low levels of SenP1 expression in the hormone refractory cell lines LNCaP-Tsai and C4-2b, and the little that exists seems to decrease with androgen withdrawal, similar to the impact that CHIP overexpression had in these cells. In contrast, androgen withdrawal upregulated SenP1 expression in hormone sensitive LNCaP cells. These results, when taken together, suggest that that SenP1 may be necessary to help LNCaP cells survive in an androgen poor environment but may not be needed by hormone insensitive cells in this same setting.

As previously noted, earlier experiments demonstrated that CHIP overexpression in LNCaP cells increased SenP1 expression and led to growth arrest (Table 1). CHIP overexpression in hormone refractory cell lines, however, decreased SenP1 expression, and these cells eventually died via autophagy. In an attempt to understand whether SenP1 or CHIP was responsible for these observations, a SenP1 knockout was made out of each of these cell lines both with and without CHIP overexpression.

Summary of CHIP Overexpression's Impact on Cell Lines			
Cell Line (Hormone sensitivity)	LNCaP Cells (Hormone sensitive)	C4-2 Cells (Hormone refractory)	LNCaP-Tsai Cells (Hormone refractory)
SenP1 Expression	INCREASED	DECREASED	DECREASED
Cell Viability	Growth Arrest	Death via Autophagy	Death via Autophagy

Table 1: Summary of the effect of CHIP overexpression on the SenP1 expression and viability of prostate cancer cell lines, LNCaP, C4-2 and LNCaP-Tsai

Consistent with previous findings, LNCaP cells overexpressing CHIP (LN-CT) appeared to undergo growth arrest while normal LNCaP cells (LN-CTD) proliferated normally. When SenP1 was knocked out, however, LNCaP cells overexpressing CHIP (LNSENP-CT) grew at rates greater than normal LNCaP cells and similar to cells with normal CHIP expression and no SenP1 (LNSENP-CTD; see Table 2).

LNCaP Cells	**SenP1 Normal**	**SenP1 Knockout**
Normal CHIP (CT)	Normal Growth	Increased growth
CHIP Over expression (CTD)	Growth Arrest	Increased growth

Table 2: The effects of CHIP expression and SenP1 expression on LNCaP cell growth

Similar SenP1 knockouts were made out of C4-2 and LNCaP-Tsai cells; these cells did not proliferate or die. These results suggested that **the loss of SenP1 may promote growth in both hormone sensitive prostate cancer cells and survival in hormone refractory prostate cancer cells that are overexpressing CHIP**. (Table 3)

Summary of CHIP Overexpression's Impact on Cell Lines			
Cell Line (Hormone sensitivity)	LNCaP Cells (Hormone sensitive)	C4-2 Cells (Hormone refractory)	LNCaP-Tsai Cells (Hormone refractory)
Downstream SenP1 Expression	INCREASED	DECREASED	DECREASED
CHIP Overexpression + Baseline SenP1	Growth Arrest	Death via Autophagy	Death via Autophagy
CHIP Overexpression + SenP1 knockout	Increased growth	Growth arrest	Growth arrest

Table 3: Summary of the impact of CHIP overexpression on SenP1 expression (row 2) and growth with normal SenP1 (row 3) and with SenP1 knocked out (row 4).

Given the differences in cell proliferation, arrest and death, we also studied CHIP's effect on Akt. The Akt and the PI3 pathway are critical in cell survival and inhibiting apoptosis. Using a Western blot staining for both Akt and Serine 473 phosphorylated Akt, we demonstrated that CHIP overexpression decreased levels of both Akt and phosphorylated Akt in LNCaP cells. When these studies were repeated in C4-2b and LNCaP-Tsai cells, there was no definitive difference in Akt expression in normal cells and those that overexpressed CHIP.

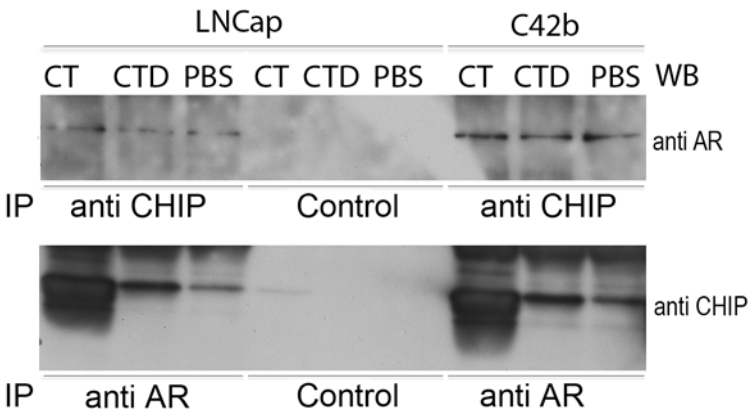

Fig. 1. **AR protein co-immunoprecipitates with CHIP protein:** CHIP (upper panel) and AR (low panel) immunoprecipitated with CHIP and AR antibodies were analyzed by Western blotting with AR antibody and CHIP antibody.

In last year's report we also described initial studies of the interaction between CHIP and the AR. Several different types of CHIP-AR interactions have been described in the literature. Studies have demonstrated that while CHIP may regulate AR levels through proteosomal degradation, there is also a component that is non-proteosomal as well. Using co-immunoprecipitation assays in both LNCaP and C4-2b cells, we demonstrated that **there is direct interaction of CHIP and AR** (figure 1). It could not be discerned from this assay, however, if CHIP overexpression has any impact on the degree or type of interaction.

We then sought to further define the interaction between CHIP and AR. Because Hsp70/Hsc70 protein is thought to play a critical role in CHIP's downstream actions and has been shown to bind both Hsp70 and Hsp90. We performed coimmunoprecipitation in both C4-2b cells and LNCaP-Tsai cells (Figure 2).

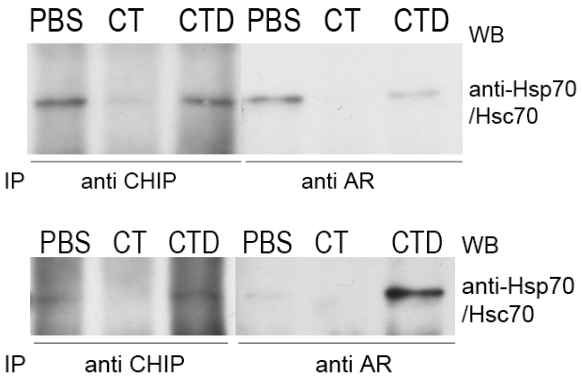

Figure 2: **AR, CHIP, and Hsp70/Hsc70 protein co-immunoprecipitate together, but only AR, CHIP co-immunoprecipitate when CHIP is overexpressed (CT)**. In LNCap C4-2B cells (upper panel) and LNCap Tsai cells (lower panel), proteins were immunoprecipitated by anti-CHIP and anti-AR, and then was analyzed by Western blotting with Hsp/Hsc 70 antibody. All cells were collected 24h after infection.

These co-immunoprecipitation experiments demonstrated that when CHIP is overexpressed (CT), Hsp70/Hsc70 were not involved in the AR-CHIP interaction. But without excess CHIP (CTD) Hsp70 was bound to both CHIP and AR.

Taken together this seems to indicate that CHIP over-expression causes death of hormone refractory prostate cancer cells, via a direct CHIP-AR interaction, as opposed to an Hsp70 associated signaling pathway.

KEY RESEARCH ACCOMPLISHMENTS

To date the following have been noted:

- CHIP-mediated loss of AR results in differential expression of several genes
- SENP1 expression is decreased in hormone independent cells overexpressing CHIP but increased in hormone sensitive cells overexpressing CHIP. This overexpression also results in death via autophagy of the androgen independent cells while it causes cell cycle arrest in hormone sensitive cells. differentially expressed both at the RNA and protein level following loss of AR in androgen sensitive versus insensitive cells
- There are no significant differences in the cell cycle regulators Cyclin E1 and Cyclin D1 in any of the cell lines, regardless of CHIP expression
- Androgen withdrawal appears to decrease SenP1 expression in hormone independent cells and increase SenP1 expression in LNCaP cells.
- SenP1 appears to control growth in different ways in androgen dependent and androgen independent cells. Without SenP1, hormone sensitive cells grew at more rapid rates, regardless of CHIP expression. But in androgen independent cells, SenP1 knockout causes growth arrest.
- Loss of SenP1 will negate the effects of CHIP overexpression in hormone sensitive cells, causing increase in growth. In hormone refractory cells, the loss of SenP1 in cells overexpressing CHIP caused what appeared to be growth arrest without death, preventing CHIP mediated death.
- In LNCaP cells, CHIP overexpression appears to decrease Akt expression levels.
- AR and CHIP interact directly both with and without CHIP overexpression in all prostate cancer cell lines tested. This interaction is not Hsp70 mediated in cells with CHIP overexpression, but Hsp70 does play a role when

REPORTABLE OUTCOMES

We believe that our work with SenP1 is compelling and worthy of publication. Additionally by characterizing the AR-CHIP interaction in milieu's both with excess CHIP and without it, we are hoping to understand CHIP's method of action on AR.

CONCLUSION

C-terminal Hsp interacting protein (CHIP) increases degradation of androgen receptor via proteosomal degradation as well as other, as yet to be characterized, pathways. CHIP and SenP1 interact both directly and indirectly. CHIP overexpression in hormone sensitive cells results in increased expression of SenP1 and Akt leading to cell cycle arrest. A different, though not opposite pattern is seen in androgen independent cells: CHIP overexpression results in decreased SenP1 expression and cell death via autophagy. There is no difference in Akt expression in hormone independent cells. Given the marked differences in expression and affect of SenP1 in hormone sensitive and independent cells, it may be a critical downstream target of CHIP.

SenP1 appears to play a critical in regulating cell growth in hormone independent and dependent cells: in hormone sensitive LnCaP cells it appears to limit growth and in hormone refractory cells it may be involved in cell death.

Additionally, there is a direct interaction between CHIP and AR. In the setting of normal CHIP expression Hsp70 may be involved however in the setting of CHIP overexpression, the interaction is independent of Hsp70 binding.

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